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14. ABSTRACT

We have investigated whether and how autoimmune complex (AIC) in SLE (lupus) can induce T helper (Th) cell polarizing cytokines including IL-27 and IL-1β, leading to enhanced Th cell responses. We have measured IL-27 gene and protein expression by healthy human monocytes g Istimulated with autoimmune complex containing anti-dsDNA and –snRNP antibodies using qPCR and ELISA, respectively. However, levels of IL-27 produced from the stimulated monocytes were relatively low compared to those of the pro-inflammatory cytokine IL-1β. IL-1β promotes of the development of Th17 cells that is increased in lupus patients. Increased IL-1β expression was found in the target tissues of lupus patients although the mechanism for these findings is unknown. It is conceivable that a combination of IL-27 and IL-1β can promote the development of Th17 cells with the capacity to produce IL-10, a notion in accordance with our original hypothesis. We have reported that AIC containing dsDNA and U1-snRNP activate human monocytes dependently of anti-dsDNA and –U1-snRNP antibodies, respectively, leading to the production of cytokines including IL-1β. This phenomenon was dependent on the activation of Toll-like receptor and NLRP3 inflammasome pathways. We also found that IL-27 can suppress IL-17 production in human CD4+ T cells and that such suppression is enhanced in patients with SLE. This finding suggests a potential therapeutic implication of exogenous IL-27 in patients with SLE.

15. SUBJECT TERMS
SLE, cytokines

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I. Introduction

We investigate a model where autoimmune complex (AIC) and IFN-alpha are driving forces for inducing cytokines that can affect T helper (Th) cells in human lupus. We have focused on the cytokine IL-27 that can enhance the development of IL-10 producing T helper (Th) 1, 2 and 17 cells as well as suppressing generation of regulatory T cells (Treg) in human lupus. In addition to IL-27, we have analyzed the proinflammatory cytokine IL-1β in parallel in that this cytokine is known to affect Th cell response in particular Th17 cells that is increased in lupus patients [1, 2]. Thus, it is conceivable that a combination of IL-27 and IL-1β can promote the development of Th17 cells with the capacity to produce IL-10, a notion in accordance with our original hypothesis. In the previous year, we reported that the production of IL-1β from AIC-activated monocytes is dependent on TLR7/8/9 and NF-κB as well as on reactive oxygen specifies (ROS) and the NLRP3 inflammasome with activate caspase-1. We published our work on IL-1β production from human monocytes by AIC of U1-snRNP and anti-U1-snRNP antibodies through activating the NLRP3 inflammasome in *The Journal of Immunology* in May 2012 [3]. We also have published our work on IL-1β production from human monocytes by AIC of dsDNA and anti-dsDNA antibodies in *The Journal of Immunology* in 2013[4]. Of interest, we have found that the cytokine IL-27 can suppress the production of IL-17 by Th cells and that such suppression is greater in lupus patients than in healthy controls (Fig 3). This finding suggests that IL-27 may reduce, rather than increase, lupus activity by suppressing the pro-inflammatory cytokine IL-17. The results of our study would advance our understanding in the lupus pathogenesis by demonstrating the role of AIC, innate immune cells, cytokines and Th cells.

II. Body

Aim 1. Investigate whether and how autoimmune complexes (AIC) containing anti-nuclear antibodies (ANA) induce IL-27 in human monocytes and DCs.

Experiment A. *Measuring IL-27 production from monocytes and myeloid (m) DCs of healthy human subjects that are incubated in the presence of 1) lupus and control sera, 2) purified immune complex (IC) containing anti-dsDNA and -snRNP Abs and 3) lupus serum depleted of total Abs, anti-dsDNA and -snRNP Abs.*

Experiment B. *Determine the role for IFN-α by adding IFN-α or anti-IFN-α Abs during the stimulations in experiment "A" above to test a role for this cytokine.*

Experiment C. *Investigate the mechanisms for AIC-mediated production of cytokines (IL-27 and IL-1β), focusing on Toll-like receptor (TLR) 7, 9 and immunoglobulin Fc receptors (FcR).*

Experiment D. *Dissect the mechanisms for AIC-mediated production of IL-27 subunits EBI3 and p28, focusing on transcriptional factor NF-κB*

The results of aim 1 were provided in annual report 2011-2012. Also, details on these results can be found in our two published papers in *the Journal of Immunology* [3, 4]. In summary, lupus autoimmune complex (AIC) hardly induced IL-27 from monocytes or mDCs (monocytes treated with IL-4 and GM-CSF). However, we found that lupus autoimmune complex (AIC) containing U1-snRNP/anti-U1-snRNP Abs or dsDNA/anti-dsDNA Abs could induce high levels of IL-1β production (1 - 10 ng/ml) from human monocytes by activating TLRs, FcγR, NF-κB and NLRP3 inflammasome. This is a very important observation. IL-1β is a potent proinflammatory cytokine primarily produced by innate immune cells such as monocytes[5, 6]. The production of IL-1β requires the activation of two pathways[7]: 1) TLR/NF-κB activation with pro-IL-1β synthesis and 2) cleaving pro-IL-1β into IL-1β by caspase-1-containing inflammasomes like the NLRP3 inflammasome. In fact, we found that lupus AIC induced the production of IL-1β from human monocytes by activating these pathways [3, 4]. Although we added IFN-α to AIC during monocyte stimulation, it did not increase IL-27 production. Our results support the findings of previous studies suggesting the potential pathogenic role of IL-1β in the pathogenesis of lupus [8-11]. Also, IL-1β can promote the development of Th17 cells[12-14], which is increased in lupus patients[1, 2, 15-17].

Aim 2. Determine whether and why AIC- and IFN-α-stimulated monocytes and DCs from lupus patients produce higher levels of IL-27 that result in enhanced development of IL-10 producing Th1, 2 and 17 cells as well as decreased generation of Treg compared to the same cells from healthy controls (timeframe, years 2-3)**.**

Experiment A. *Compare production of IL-27, EBI3 and p28 from monocytes and mDCs of healthy controls, mild and severe lupus patients* by incubating the cells in the presence of AIC and IFN-α and analyzing them as in Experiment A of Aim 1.

As previously reported (annual report 2011-12), we found that lipopolysaccharide (LPS) or combination of LPS and IFN-γ induced high levels of IL-27 from monocytes although AIC hardly induced IL-27 from monocytes and mDCs. We have stored cell culture supernatants and lysates of peripheral blood mononuclear cells that were stimulated with IFN-γ, LPS or both in patients with SLE (n = 33) and healthy controls (n = 18). PBMCs contain both monocytes and mDCs. We will measure IL-27, EBI3 and p28 gene and protein to determine whether lupus patients have any alteration in IL-27 production using qPCR and ELISA as appropriate during this coming year. We also measured the expression of IFN-γ receptor 1 and 2 on monocytes of lupus patients and healthy controls. Of interest, we have found that lupus monocytes have increased expression of IFN-γR1 compared to healthy monocytes (Fig 1). A similar trend was observed for IFN-γR2 although it did not reach the level of statistical significance. These findings suggest that lupus patients may have increased IL-27 production in response to IFN-γ secondary to increased IFN-γR1 and 2 expression.

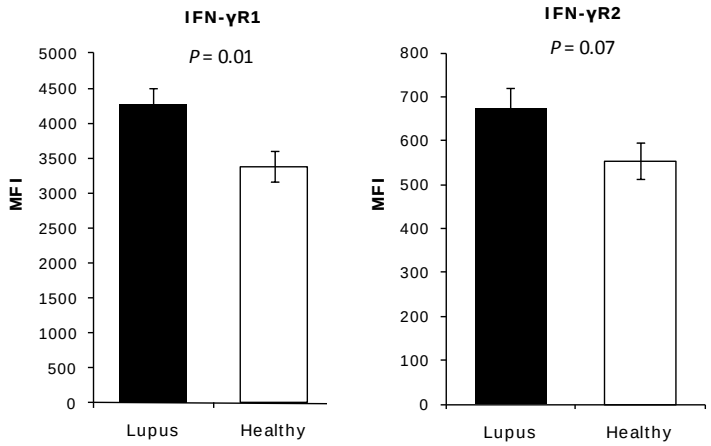


Figure 1. The expression of IFN-γR1 and 2 on monocytes in lupus patients and healthy controls. PBMCs from lupus patients (n = 23) and healthy controls (n =15) were stained with antibodies to CD14, IFN-γR1, 2 or isotype control. Stained cells were analyzed on a flow cytometer. The mean fluorescent intensity (MFI) of IFN-γR1 and 2 expression on CD14+ monocytes was determined by subtracting MFI of isotype staining from MFI of IFN-γR1 or 2. Bars indicate mean and standard error of mean. *P* value was obtained by unpaired *t*-test.

Experiment B. *Correlate the production of IL-27 from monocytes and mDCs with serum levels of IL-27, IL-10, IFN-α and disease activity (SLEDAI) as well as the frequency of Th cells producing IL-10 and Treg.*

As reported last year, we have stimulated PBMCs from patients with SLE and healthy controls for 4 hours with PMA/ionomycyin to determine the frequency of Th cells producing cytokines including IL-10. In this experiment, we have found that the frequency of IL-10-producing cells was low (typically less than 0.5%). However, IL-17- and/or IFN-γ-producing CD4+ T cells as well as FOXP3+ Treg cells have been constantly found in both groups. Details on statistical analysis of this experiment are found in aim 3. We will measure cytokine levels in the circulation using stored plasmas. These findings will be correlated with the results of flow cytometric analysis and clinical data (disease activity).

Experiment C*. Investigate possible mechanisms for altered IL-27 production in lupus patients, focusing on transcriptional factors NF-κB and IRF-1.*

We demonstrated the role of NF-κB in activating human monocytes with lupus AIC as measured by IL-1β production from AIC-treated monocytes in the presence or absence of NF-κB inhibitors. Also, we showed the activation of NF-κB in human monocytes by AIC. These are found in our published papers (references 3 and 4;

see also Appendix) and previous report. Of interest, we hardly detected IL-27 production from similarly treated monocytes, suggesting no role of AIC in inducing IL-27. Since LPS or a combination of LPS and IFN-γ can induce IL-27 production, we will measure IRF1, which is known to be up-regulated by IFN-γ and LPS, from LPS- and/or IFN-γ-treated PBMCs from Exp A of Aim 2 if we find a difference in the production of IL-27 from PBMCs in response to IFN-γ and/or LPS.

Experiment D. *Determine whether lupus monocytes and mDCs are better in inducing IL-10 producing Th cells and suppressing FOXP3 expressing cells via IL-27 compared to healthy controls.*

We found that cell culture supernatants of monocytes treated with lupus AIC produced high levels of IL-1β, but not IL-27, with the capacity to promote IL-17 production from CD4+ T cells. Thus, we studied whether such supernatants could enhance IL-17 production from CD4+ T cells. In the presence of culture supernatant from monocytes treated with dsDNA and anti-dsDNA Abs, the levels of IL-17 production from CD4+ T cells with the expression of IL-1 receptor 1 were much higher than in the absence of this supernatant (Fig 2). The increase was blocked by adding IL-1 receptor antagonist that blocks the binding of IL-1β to IL-1 receptor. A role for IL-17 in lupus pathogenesis has been suggested by recent studies showing enhanced antibody production from B cells by IL-17 and increased Th17 cell response in lupus patients. Our finding provides a possible mechanism for the increased Th17 response in human lupus.

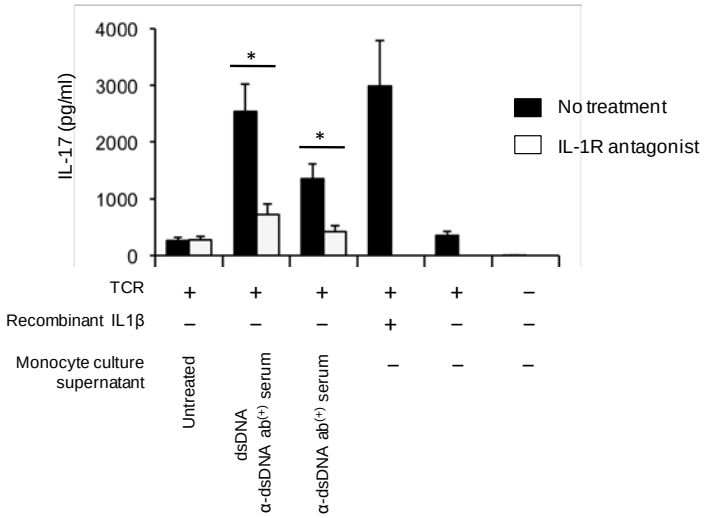


Fig 2. Cell culture supernatant from monocytes treated with a combination of self dsDNA and anti-dsDNA antibody-positive serum promotes IL-17 production from IL-1 receptor I (IL-1RI)-positive memory CD4+ T cells in an IL-1β-dependent manner. ELISA of IL-17 production from sorted human IL-1RI+ memory CD4+ T cells that were stimulated for 5 days with anti-CD3 and -CD28 antibody-coated beads in 80% culture medium with 20% of supernatants from monocytes treated for 18 hours with self dsDNA in the presence or absence of anti-dsDNA antibody-positive serum. Some IL-1RI+ memory CD4+ T cells were incubated with culture medium alone or medium with anti-CD3 and anti-CD28 antibody-coated beads in the presence or absence of recombinant IL-1β (20 ng/ml). IL-1R antagonist (100 ng/ml) was added on days 0 and 2. Bars and error bars indicate mean and SEM, respectively (n = 5). The presence and absence of each treatment are indicated by + and −, respectively. *P < 0.05.

Aim3. Investigate whether enhanced development of IL-10 producing Th1, 2 and 17 cells as well as decreased generation of Treg in lupus are secondary to alteration(s) in IL-27 receptor (IL-27R) expression and signaling in CD4+ T cells (timeframe, years 2-3)**.**

Experiment A. *Determine IL-27-mediated generation of IL-10 producing Th1, 2 and 17 cells as well as FOXP3 expressing Treg in lupus patients and healthy controls.*

We have treated PBMCs from lupus patients and healthy controls for 16 hours with IL-27 in the presence of T cell stimulus (PMA/ionomycin and anti-CD3/CD28 Abs, respectively). Although 16 hour stimulation minimally increases IL-10 production from CD4+ T cells, it suppressed IFN-γ and IL-17 production. Of interest, IL-27 could modestly increase FOXP3 in CD4+ T cells (approximately by 10% from the baseline). In order to determine whether lupus patients have any alteration in these responses to IL-27, we have continued to measure the frequency of CD4+ T cells producing IL-17, IFN-γ and IL-10 in lupus patients and healthy controls. In our analysis, CD4+ T cells from lupus patients had enhanced response to IL-27 treatment compared to CD4+ T cells from healthy controls. The IL-27-mediated decrease in the frequency of IL-17-producing CD4+ T cells was much greater in lupus patients than in healthy controls (see Figure 3, please note that the frequency of Th17 cells is typically less than 1% of total CD4+ T cells in healthy controls while it is about 2% in lupus patients). However, the IL-27-mediated decrease in the frequency of IFN-γ-producing CD4+ T cells

was similar between lupus patients and healthy controls (see Figure 3). There was no difference in the IL-27-mediated change in the frequency of IL-10-producing CD4+ T cells between the two groups. These are intriguing findings. Our group previously reported that lupus patients had increased Th17 cell response that correlated with disease activity (Shah *et al.* 2010. *Arthritis Research & Therapy*). Thus, IL-27 could be considered a possible therapeutic agent to reduce IL-17 production in lupus patients in that its inhibitory effect on Th17 cells is much greater in lupus patients compared to healthy controls while the effect of IL-27 on IFN-γ and IL-10 is similar.

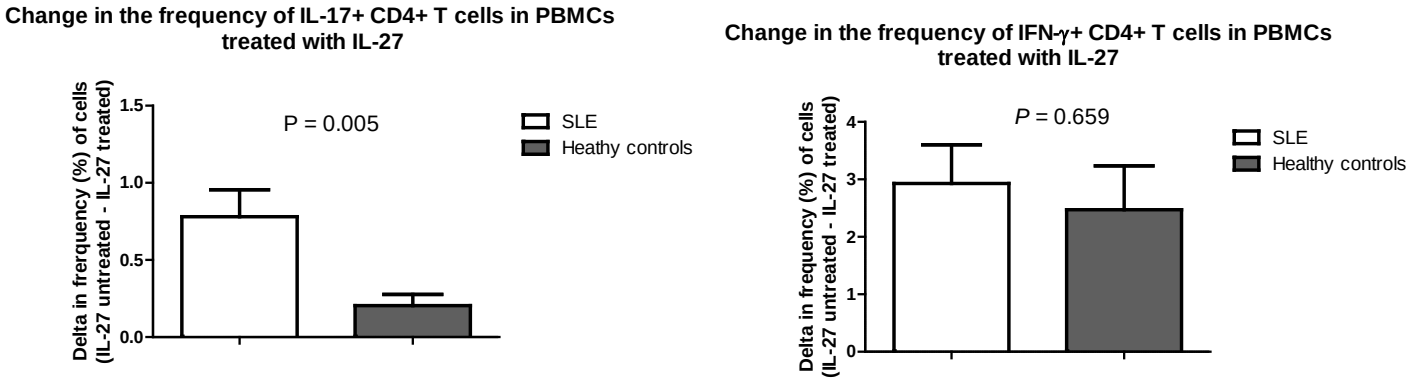
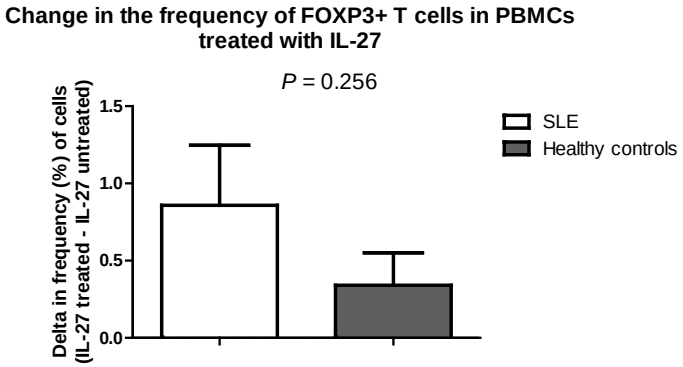


Figure 3. The IL-27-mediated decrease in the frequency of IL-17- but not IFN-γ-producing CD4+ T cells is greater in lupus patients than in healthy controls. PBMCs from lupus patients (n = 21) and healthy controls (n =11) were incubated for 16 hours in the presence or absence of IL-27 (50 ng/ml). During the last four hours of incubation, PMA/ionomycin and Golgi plug was added. IL-17-producing CD4+ T cells were identified by flow cytometry. The change (%) in the frequency of IL-17- or IFN-γ-producing CD4+ T cells between IL-27-treated and –untreated samples was calculated. Bars and error bars indicate mean and standard error of mean. *P* value was obtained by unpaired *t*-test.



In terms of FOXP3 expression, IL-27 increased the expression of FOXP3 in CD4+ T cells although the difference of this increase was not different between lupus patients and healthy controls (Fig 4).

Figure 4. The IL-27-mediated increase in the frequency of FOXP3+ CD4+ T cells is not different in lupus patients and healthy controls. PBMCs from lupus patients (n = 21) and healthy controls (n =11) were incubated for 16 hours in the presence or absence of IL-27 (50 ng/ml). FOXP3+ CD4+ T cells were identified by flow cytometry. The change in the frequency(%) of FOXP3+ CD4+ T cells between IL-27-treated and –untreated samples was calculated. Bars and error bars indicate mean and standard error of mean. *P* value was obtained by unpaired *t*-test.

Experiment B. *Determine the mechanism for altered IL-27 response in lupus CD4+ T cells (experiment A) by measuring IL-27 receptor (R) expression and signaling in CD4+ T cell subsets in lupus patients and healthy controls.*

In aim 3, we have continued measuring the IL-27 receptor (WSX-1) and its signaling unit gp130 on human $CD4^+$ T cells in lupus patients and healthy controls to determine whether lupus CD4+ T cells have any alteration in such cytokine receptor expression, leading to increased response to IL-27. The results of this analysis showed no difference in the expression of IL-27 receptor and gp130 on CD4+ T cell subsets between lupus patients and healthy controls (Figure 5). These findings suggest that the exaggerated effect of IL-27 on IL-17-producing CD4+ T cells is unlikely secondary to increased IL-27R expression on CD4+ T cells.

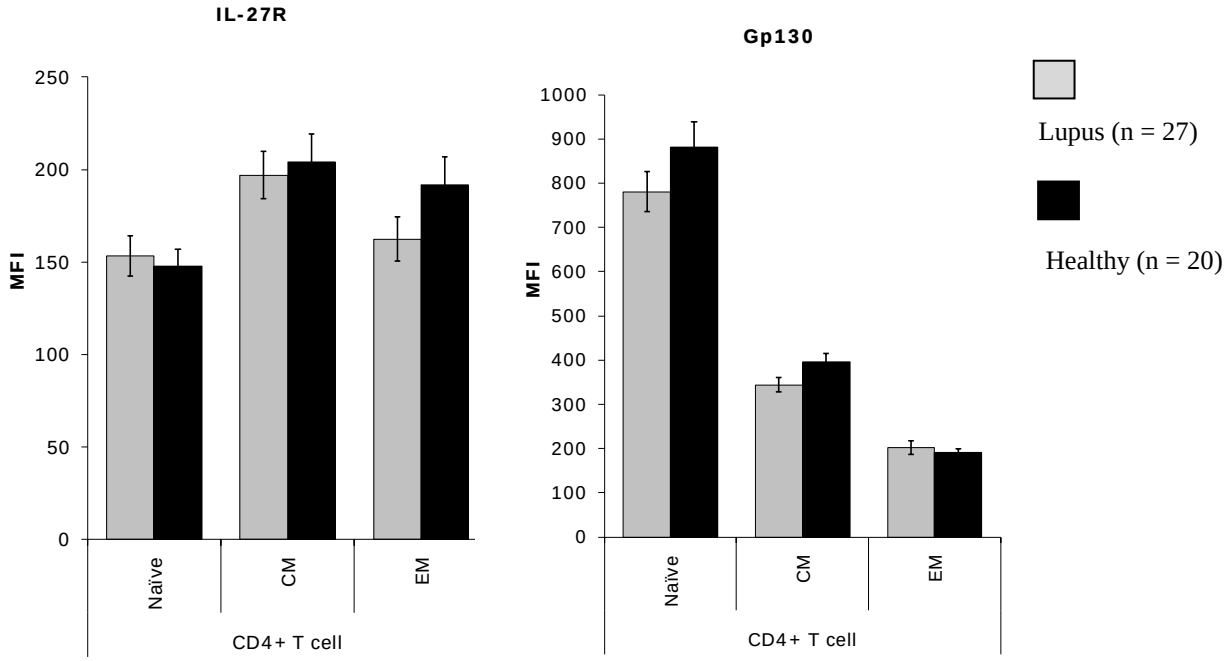


Figure 4. The expression levels of IL-27 receptor (R) and gp130 on CD4+ T cell subsets are similar in lupus patients and healthy controls. PBMCs from lupus patients (n = 27) and healthy controls (n = 20) were stained with CD3, CD4, CD45RA, CCR7, IL-27R, gp130 or isotype. Stained cells were analyzed on an LSRII flow cytometer. The mean fluorescent intensity (MFI) of IL-27 and gp130 expression was measured on naïve (CD45RA+CCR7+), central memory (CM, CD45RA-CCR7+) and effector memory (EM, CD45RA-CCR7-) CD4+ T cells. *P* values were greater than 0.05 as measured by unpaired t-test.

Aim 4. Prospectively determine the relationship of IL-27 production with disease parameters in lupus patients as well as in biopsies of their cutaneous lesions (timeframe, years 1-3).

Experiment A. *Prospectively measure and correlate the following parameters with disease activity of lupus (SLEDAI).*

We have prospectively collected blood samples from a total of 8 lupus subjects who donated blood between 2-4 times. We have stored plasmas and cell pellets as well as analyzed Th cell responses to IL-27. We will analyze these samples and correlate with disease activity during this coming year (no cost extension year).

Experiment B. *Determine the expression of IL-27, IL-10, IFN-α and FOXP3 in lupus cutaneous lesions using immunohistochemial staining.*

As reported last year, we measured IL-1β and caspase-1 expression by CD14+ cells in lupus cutaneous lesions since we could not detect substantial production of IL-27 from lupus autoimmune complex (AIC)-stimulated human monocytes in Aim 1. Also, the frequency of IL-10-producing CD4+ T cells was quite low. However, we noticed the presence of $CD14^+$ cells like monocytes which expressed caspase-1 and IL-1β in the acute cutaneous lupus lesion (please see Fig 9 in annual report 2011-2012). These findings further support the potential role of IL-1β and caspase-1 activation in the pathogenesis of lupus.

III. Key Research Accomplishments

● Lupus AICs (autoimmune complexes dsDNA/anti-dsDNA antibodies and U1-snRNP/anti-U1-snRNP antibodies) induce the production of IL-1β but not IL-27 from human monocytes by activating TLR7/8/9, NF-κB and NLRP3 inflammasome.

● *Ex vivo* IL-27 treatment suppresses the production of IL-17, which is increased in lupus patients, by CD4+ T cells, and such suppression is greater in lupus patients than in healthy controls.

● Lupus patients and healthy controls have similar levels of IL-27 receptor expression on CD4+ T cell subsets, suggesting that enhanced IL-27-mediated response in lupus patients is unlikely related to altered IL-27 receptor expression.

● IFN-γ alone or a combination of IFN-γ and LPS could induce IL-27 from human PBMCs, and monocytes of lupus patients have increased IFN-γ receptor 1 expression.

IV. Reportable Outcomes

We have published a manuscript entitled "Self Double-Stranded (ds)DNA Induces IL-1β Production from Human Monocytes by Activating NLRP3 Inflammasome in the Presence of Anti–dsDNA Antibodies" in *the Journal of Immunology* in 2013 (see attached manuscript).

V. Conclusion

The results of our study show that lupus autoimmune complex (AIC) containing dsDNA and U1-snRNP can induces the production of IL-1β but not IL-27 from human monocytes by activating TLR7/8/9 and NLRP3 inflammasome. Such IL-1β production could enhance the production of IL-17 from CD4+ T cells which is known to be increased in lupus patients [1, 14, 15, 17-19]. Thus, our findings provide a potential mechanistic explanation for the increased Th17 cell response in lupus patients. Also, we have found that IL-27 decreases IL-17. Of interest, the IL-27-mediated suppression of IL-17 production by CD4+ T cells was greater in lupus patients than in healthy controls. These findings suggest a possible therapeutic role of IL-27 in lupus by suppressing IL-17 production from lupus patients.

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VII. Appendices

Published manuscript: Shin, M. S. et al. Self Double-Stranded (ds)DNA Induces IL-1beta Production from Human Monocytes by Activating NLRP3 Inflammasome in the Presence of Anti-dsDNA Antibodies. J Immunol 190, 1407-15 (2013)

Self Double-Stranded (ds)DNA Induces IL-1β Production from Human Monocytes by Activating NLRP3 Inflammasome in the Presence of Anti–dsDNA Antibodies

Min Sun Shin,* Youna Kang,* Naeun Lee,* Elizabeth R. Wahl,* Sang Hyun Kim,*,† Ki Soo Kang,*,‡ Rossitza Lazova,§ and Insoo Kang*

The pathogenic hallmark of systemic lupus erythematosus is the autoimmune response against self nuclear Ags, including dsDNA. The increased expression of the proinflammatory cytokine IL-1β has been found in the cutaneous lesion and PBMCs from lupus patients, suggesting a potential involvement of this cytokine in the pathogenesis of lupus. IL-1β is produced primarily by innate immune cells such as monocytes and can promote a Th17 cell response, which is increased in lupus. IL-1β production requires cleaving pro–IL-β into IL-1β by the caspase-1–associated multiprotein complex called inflammasomes. In this study we show that self dsDNA induces IL-1β production from human monocytes dependent on serum or purified IgG containing anti–dsDNA Abs by activating the nucleotide-binding oligomerization domain–like receptor family pyrin domain–containing 3 (NLRP3) inflammasome. Reactive oxygen species (ROS) and K^+ efflux were involved in this activation. Knocking down the *NLRP3* or inhibiting caspase-1, ROS, and K^+ efflux decreased IL-1β production. Supernatants from monocytes treated with a combination of self dsDNA and anti–dsDNA Ab^+ serum promoted IL-17 production from $CD4^+$ T cells in an IL-1β–dependent manner. These findings provide new insights in lupus pathogenesis by demonstrating that self dsDNA together with its autoantibodies induces IL-1β production from human monocytes by activating the NLRP3 inflammasome through inducing ROS synthesis and K^+ efflux, leading to the increased Th17 cell response. ***The Journal of Immunology*****, 2013, 190: 1407–1415.**

The innate immune cells such as monocytes, macrophages, and dendritic cells provide the first line of defense against microorganisms. These cells are armed with the germ line–encoded pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) commonly found in microorganisms (1, 2). Different classes of PRRs have been identified. These receptors include TLRs, retinoic acid–inducible gene-I–like receptors, nucleotide-binding oligomerization domain–like receptors (NLRs), and absent in melanoma 2 (AIM2) (1–3). TLRs that exist on the cell surface or within the intracellular vesicular compartments, such as endosomes and lysosomes, recognize PAMPs present outside of cells or delivered into these compartments (1). Retinoic acid–inducible gene-I–like receptors, NLRs, and AIM2, which are located in the cytosol, can detect PAMPs within the cytosol (1, 3).

Inflammasomes are multimeric protein complexes with the capacity to activate the caspase-1 that cleaves pro–IL-1β into IL-1β (2, 4). Different types of inflammasomes contain distinct PRRs responsible for the activation of the inflammasomes. For instance, the NLR family pyrin domain–containing 3 (NLRP3) is associated with the NLRP3 inflammasome whereas AIM2 is found in the AIM2 inflammasome (2, 4). An array of molecules from host and environments as well as from microorganisms has been reported as inflammasome activators. AIM2 inflammasome is activated by cytosolic dsDNA from host and pathogens through its binding to the C-terminal HIN domain of AIM2 (5, 6). Activators of the NLRP3 inflammasome are heterogeneous, ranging from self-originating uric acid, calcium pyrophosphate crystals, cholesterol crystals, ATP, and glucose to environment-derived aluminum hydroxide, silica, and asbestos as well as molecules from pathogens (reviewed in Refs. 2, 4). Although it is yet to be determined how molecules with such diverse structures could activate the NLRP3 inflammasome, reactive oxygen species (ROS) and K^+ efflux appear to be important mediators for the activation of the NLRP3 inflammasome (7).

Systemic lupus erythematosus (SLE or lupus) is an autoimmune inflammatory disease of unknown etiology that affects multiple organs, including the joint, skin, kidneys, and hematologic system (8). The immunologic hallmark of lupus is autoantibodies against nuclear proteins and dsDNA. In particular, anti–dsDNA Abs and circulating dsDNA/anti–dsDNA immune complexes are found in lupus patients (9, 10). A correlation of disease activity with titers of anti–dsDNA Abs has been found in lupus patients (11, 12), suggesting a pathogenic role of these Abs. In fact, the immune stimulatory property of dsDNA has been reported (10, 13–18). In

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Abbreviations used in this article: AIM2, absent in melanoma 2; ANA, antinuclear Ab; DPI, diphenyleneiodonium; NLR, nucleotide-binding oligomerization domain–like receptor; NLRP3, nucleotide-binding oligomerization domain–like receptor family pyrin domain-containing 3; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; PRR, pattern recognition receptor; qPCR, quantitative PCR; ROS, reactive oxygen species; SLE, systemic lupus erythematosus.

the presence of anti–dsDNA Abs, self dsDNA stimulated B cells and plasmacytoid dendritic cells (pDCs) dependent on TLR9, leading to increased Ab and IFN-α production (10, 13, 14, 17). Additionally, dsDNA from self and non-self could activate cytosolic AIM2 inflammasome in innate immune cells and keratinocytes when the cells were infected with virus or transfected with plasmid or host DNA in the presence of DOTAP (Roche, Indianapolis, IN) (5, 6, 18–20). The production of IL-1β from the THP-1 cells and murine macrophages infected with adenovirus, a non-enveloped DNA virus, was dependent in part on the NLRP3 inflammasome, suggesting an activation of this inflammasome by DNA (21). Of interest, increased IL-1β gene or protein expression was found in the PBMCs and skin lesions of lupus patients (22, 23). Similarly, the *ilb* gene was detected in the nephritis tissues from lupus-prone mice (24–26). Additionally, Th17 cell response, which is promoted by IL-1β, was increased in lupus patients (27–31). These observations raise the potential involvement of IL-1β and inflammasomes in the pathogenesis of lupus.

In the current study, we investigated whether and how self dsDNA, a molecular target of autoimmune responses in lupus, could induce IL-1β production from human monocytes, a major cellular source of IL-1β. Our results show that self dsDNA can induce IL-1β production from human monocytes in the presence of anti–dsDNA Abs by activating the NLRP3 inflammasome. ROS and K^+ efflux were responsible for this activation. Knocking down the *NLRP3* or inhibiting ROS, K^+ efflux, caspase-1, or the TLR9 pathway decreased IL-1β production. Additionally, supernatants from monocytes treated with dsDNA and anti–dsDNA Ab^+ serum promoted IL-17 production from $CD4^+$ T cells in an IL-1β–dependent manner. The results of our study indicate the potential role of IL-1β and NALP3 inflammasome in the pathogenesis of lupus by demonstrating that dsDNA together with its autoantibodies induces IL-1β production from human monocytes by activating the NLRP3 inflammasome through ROS synthesis and K^+ efflux, leading to the increased Th17 response.

Materials and Methods

Human monocytes and sera

This work was approved by the Institutional Review Committee of Yale University. Peripheral blood was obtained from the New York Blood Center or healthy adults after obtaining informed consent. Fresh monocytes were isolated from PBMCs using a negative cell purification kit (StemCell Technologies, Vancouver, BC, Canada). Antinuclear Ab (ANA)–positive sera with or without anti–dsDNA Abs were obtained from L^2 Diagnostics laboratories. The presence of ANA and anti–dsDNA Abs were determined by indirect immunofluorescence assay using Hep2 cells and *Crithidia luciliae*, respectively. Healthy human sera were obtained from peripheral blood of healthy adult donors.

Monocyte stimulation

Monocytes were resuspended in RPMI 1640 media supplemented with 10% FCS, penicillin, and streptomycin at 5×10^5 cells/ml. Human genomic dsDNA was isolated from Jurkat and THP-1 cell lines using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). Monocytes were stimulated with or without human genomic dsDNA (5 μg/ml) in the presence or absence of serum (5% final concentration) or total IgG with or without anti–dsDNA Abs. Total IgG was purified from sera using a NAb spin kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. To deplete anti–dsDNA Abs from Ab^+ serum, serum was diluted with RPMI 1640 media (5% final concentration) and incubated overnight at 4°C in a sterile ELISA plate coated with dsDNA (20 μg/ml). After the incubation, serum was collected and analyzed for anti–dsDNA Abs by ELISA to determine levels of depletion. In some experiments, monocytes were additionally treated with the following: ant–CD32 (FcγRII) Abs (2.5 μg/ml; R&D Systems, Minneapolis, MN) (10), NF-κB inhibitors (5 μM Bay11-7082 and 5 μM celastrol; InvivoGen, San Diego, CA), caspase-1 inhibitor (5 μM Ac-YVAD-CMK; Enzo Life Sciences International, Plymouth Meeting, PA), diphenyleneiodonium (50 μM DPI; Sigma-Aldrich), chloroquine (5 μg/ml; Sigma-Aldrich), scavenger receptor blockers (20 μM tannic acid and 50 μg/ml dextran sulfate; Sigma-Aldrich), and heparin (50 U; Baxter, Deerfield, IL) (32–34) or inhibitory nucleic acid sequence for TLR9 (oligodeoxynucleotide, 5 μM final concentration) (17). The TLR inhibitory sequence was preincubated for 15 min with DOTAP (Rooche) at room temperature before adding to monocytes.

Purification and stimulation of IL-1RI$^+$ memory CD4$^+$ T cells

Human IL-1RI$^+$ memory ($CD45RA^-$) $CD4^+$ T cells were purified from PBMCs of healthy donors using a FACSAria as previously described (35). Purified cells were stimulated for 5 d with anti–CD3 and anti–CD28 Ab-coated beads in RPMI 1640 medium mixed at a 4:1 ratio with supernatants from stimulated monocytes in the presence or absence of IL-1R antagonist (100 ng/ml; R&D Systems). IL-1R antagonist was added again on day 2. Some IL-1RI$^+$ memory $CD4^+$ T cells were treated with culture medium alone or with anti–CD3 and anti–CD28 Ab-coated beads in the presence or absence of human rIL-1β (20 ng/ml; R&D Systems).

Measuring NF-κB activation

Monocytes were incubated for 4 h with or without human genomic dsDNA in the presence or absence of healthy serum or anti–dsDNA Ab^+ serum (5% final concentration). This time point was determined based on the results of a kinetic study (Supplemental Fig. 2A). Following fixation and permeabilization, cells were stained with anti–phosphorylated NF-κB p65 Abs (pS529; BD Biosciences, San Diego, CA). Cells were analyzed on a flow cytometer.

Measuring caspase-1 activation and ROS synthesis

Monocytes were incubated for 7 h (caspase-1) or for 3–4 h (ROS) with or without healthy serum, anti–dsDNA Ab^+ serum (5% final concentration), or IgG purified from anti–dsDNA Ab^+ serum in the presence or absence of human genomic dsDNA. The active caspase-1 was detected by flow cytometry using FAM FLICA caspase-1 assay kit (Immunochemistry Technologies, Bloomington, MN) according to the manufacturer's instructions. To measure ROS synthesis, stimulated cells were added with ROS detection reagent 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (C400; Invitrogen) at the end of stimulation. Cells were then analyzed on a flow cytometer. The incubation time points were selected based on the results of kinetic studies (Supplemental Fig. 2B, 2C).

Knocking down the NLRP3 gene

Human monocytes were nucleofected with scrambled siRNA or *NLRP3*-specific siRNA (Invivogen) using the Amaxa Nucleofector system and a human monocyte Nucleofector kit (Lonza, Walkersville, MD) according to the manufacturer's instruction. Following 6 h resting, nucleofected cells were incubated for 18 h with or without dsDNA and anti–dsDNA Ab^+ serum. Knockdown of the *NLRP3* gene was determined by Western blotting with anti–human NLRP3 Ab (Enzo Life Sciences) and quantitative PCR (qPCR) with the following primers: forward, 5′-CCACAAGATCGTGAGAAAACCC-3′, reverse, 5′-CGGTCCTATGTGCTCGTCA-3′.

ELISA and qPCR

IL-1β and IL-18 from monocytes and IL-17 from $CD4^+$ T cells in culture supernatants were measured by ELISA (eBioscience, San Diego, CA). Pro-*IL1B* gene and pro–IL-1β protein in cell lysate were measured using real-time qPCR and a human pro–IL-1β ELISA kit (R&D Systems), respectively. Briefly, total RNA was extracted from cells using RNeasy Plus Midi kit (Qiagen) and cDNA was synthesized. Real-time qPCR was done on an Mx3005P qPCR system (Stratagene, La Jolla, CA) using the 2X Brilliant SYBR Green Master mix (Stratagene). The primer sequences for the *IL1B* gene were: forward, 5′-CACGATGCACCTGTACGATCA-3′, reverse, 5′-GTTGCTCCATATCCTGTCCCT-3′.

The levels of gene expression were normalized to the expression of *ACTINB* (35). Also, the *NLRP3* gene in monocytes was measured by qPCR using the primer sequences as described above. The comparative C_T method ($\Delta\Delta C_T$) was used for quantification of gene expression.

Statistical analysis

Paired *t* tests were performed for statistical analysis using Microsoft Excel. A p value <0.05 was considered statistically significant.

Results

Self dsDNA induces IL-1β production from human primary monocytes in the presence of anti–dsDNA Abs

dsDNA from self and non-self could activate cytosolic AIM2 inflammasome in innate immune cells and keratinocytes when the cells were infected with virus or transfected with plasmid or host DNA in the presence of DOTAP (5, 6). We investigated whether human dsDNA alone could induce IL-1β production from human monocytes, which are a primary cellular source of this cytokine (36). dsDNA alone could not induce IL-1β production (Fig. 1). However, dsDNA induced IL-1β production from monocytes in the presence of serum containing anti–dsDNA Abs (Fig. 1). IL-1β production was not detected from monocytes treated with dsDNA in the presence of healthy serum or serum positive for ANA but negative for anti–dsDNA Abs (Fig. 1C). Similarly, human monocytes produced IL-1β in response to dsDNA in the presence of IgG purified from anti–dsDNA Ab^+ serum (Fig. 2A). Next, we selectively depleted anti–dsDNA Abs from the Ab^+ serum by incubating the serum overnight in a plate coated with self dsDNA. Although we depleted these Abs only partially (data not shown), it still decreased IL-1β production from monocytes treated with dsDNA (Fig. 2B). A previous study reported that immune complexes containing dsDNA bound the IgG receptor CD32 (FcgRII) on pDCs (10). Thus, we blocked CD32 on monocytes with anti–CD32 Abs, which significantly reduced IL-1β production (Fig. 2C). These data indicate that dsDNA induces IL-1β production from human monocytes in an anti–dsDNA Ab-dependent manner. Of interest, monocytes incubated with anti–dsDNA Ab^+ serum or IgG alone also produced IL-1β (Figs. 1, 2A). However, the levels of IL-1β produced from such treated cells were lower than those produced from monocytes treated additionally with dsDNA. These findings suggest the presence of a complex of dsDNA and anti–dsDNA Abs in anti–$dsDNA^+$ sera as previously reported (9, 10). Indeed, treating anti–dsDNA Ab^+ serum with DNase reduced IL-1β production from monocytes (Fig. 2D). Because anti–CD32 Abs or DNase partially reduced IL-1β production from monocytes treated with dsDNA and anti–dsDNA Ab^+ serum, we considered scavenger receptor A as an alternative pathway for such IL-1β production. Previous studies reported the involvement of scavenger receptors in the uptake of nucleic acids and Ags into cells (32, 37). We found the expression of scavenger receptor A on human monocytes and decreased IL-1β production from monocytes treated with dsDNA and anti–dsDNA Ab^+ serum in the presence of known scavenger receptor A inhibitors, including tannic acid, dextran sulfate, and heparin (Supplemental Fig. 1A, 1B) (32–34).

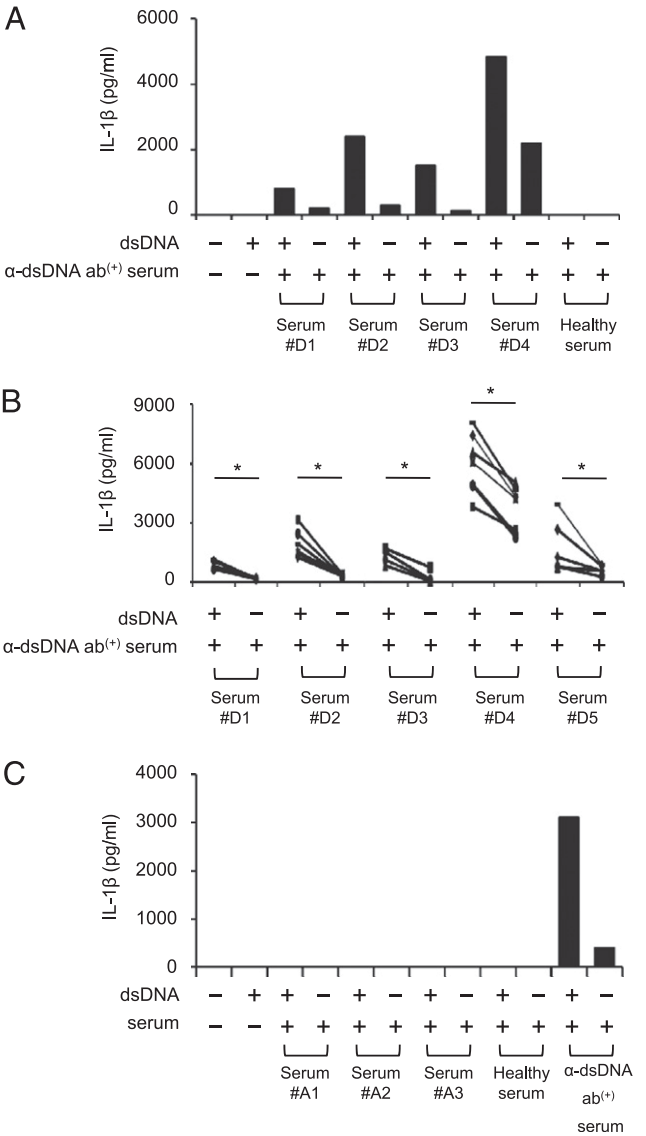

FIGURE 1. Human monocytes produce IL-1β in response to self dsDNA in the presence of anti–dsDNA Ab^+ serum. (**A**–**C**) Measuring IL-1β in cell culture supernatants of monocytes incubated for 18 h in the following conditions by ELISA. (A and B) Monocytes purified from a single (A) or multiple healthy donors (B, symbols indicate individual donors) were incubated with human genomic dsDNA (5 μg/ml) in the presence or absence of healthy serum or anti–dsDNA Ab^+ serum (5% final concentration) from multiple donors (A, numbers [nos.] D1–D4; B, nos. D1–D5). (C) ANA^+ sera without anti–dsDNA Abs (donors, nos. A1–A3) were added to monocytes from a single donor in the presence or absence of dsDNA. Representative data are from two independent experiments (A and C). *$p < 0.05$.

NF-κB and TLR9 are involved in producing IL-1β from human monocytes in response to a combination of self dsDNA and anti–dsDNA Abs

The production of IL-1β is tightly regulated at the transcriptional and posttranslational levels through NF-κB and inflammasome activations, respectively (1). Triggering TLRs activates NF-κB, leading to increased synthesis of pro–IL-1β. Pro–IL-1β is then cleaved into the active form IL-1β by caspase-1 containing inflammasomes and secreted outside cells. Thus, we determined whether human monocytes stimulated with dsDNA and its Abs had increased generation of pro–IL-1β gene and protein. Pro-*IL1B* transcripts were highly expressed in monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum although they were barely detected in monocytes treated with dsDNA alone or a combination of dsDNA and healthy serum (Fig. 3A). The same results were found when we measured pro–IL-1β protein in lysates from monocytes treated with dsDNA or a combination of dsDNA and anti–dsDNA Ab^+ serum (Fig. 3B).

We next measured NF-κB activation in human monocytes treated with dsDNA in the presence or absence of anti–dsDNA Ab^+ serum or healthy serum. NF-κB was highly activated in monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum but not in monocytes incubated with dsDNA alone or with healthy serum in the presence or absence of dsDNA (Fig. 4A, Supplemental Fig. 2A). Although the activation of NF-κB was found in monocytes treated with anti–dsDNA Ab^+ serum alone, the levels of such activation were lower than those in monocytes treated additionally with dsDNA. Furthermore, IL-1β production from monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum or anti–dsDNA Ab^+ serum alone was blocked

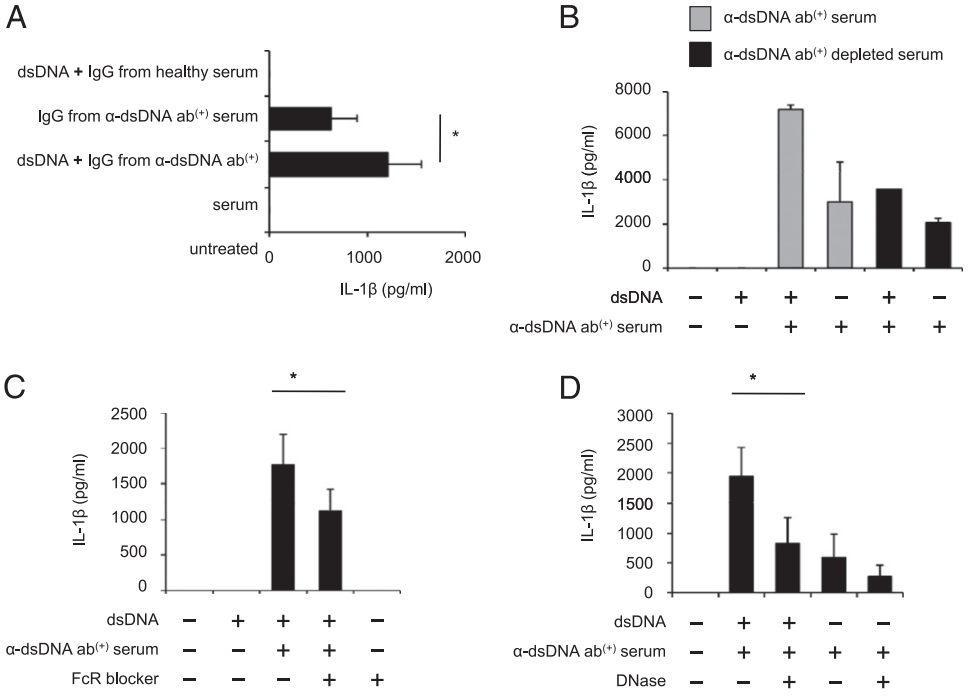

FIGURE 2. Anti–dsDNA Abs are required for IL-1β production from human monocytes treated with self dsDNA. (**A**–**D**) Measuring IL-1β in cell culture supernatants of monocytes incubated for 18 h in the following conditions by ELISA. (A) Monocytes from healthy donors were incubated with dsDNA in the presence or absence of total IgG purified from anti–dsDNA Ab^+ serum or healthy serum ($n = 5$). (B) Monocytes were incubated with dsDNA in the presence or absence of serum depleted or undepleted of anti–dsDNA Abs (two independent experiments). (C) Monocytes were treated with anti–CD32 Abs (FcR blocker, 2.5 μg/ml) and incubated with dsDNA and anti–dsDNA Ab^+ serum ($n = 5$). (D) Monocytes were incubated with anti–dsDNA Ab^+ serum or dsDNA and anti–dsDNA Ab^+ serum in the presence or absence of DNase (1 μg/ml) ($n = 7$). Bars and error bars indicate mean and SEM, respectively. *$p < 0.05$.

by NF-κB inhibitors (Fig. 4B, Supplemental Fig. 3). DNA is known to trigger TLR9 in the endosome, leading to NF-κB activation (1). Indeed, human monocytes expressed high levels of TLR9 (data not shown). The endosomal inhibitor chloroquine and inhibitory nucleic acid sequence for TLR9 significantly reduced IL-1β production from moncytes treated with dsDNA and anti–dsDNA Ab^+ serum (Fig. 4C, 4D, Supplemental Fig. 3). Our observations indicate the essential role of TLR9 and NF-κB in producing IL-1β by human monocytes in response to self dsDNA and anti–dsDNA Abs.

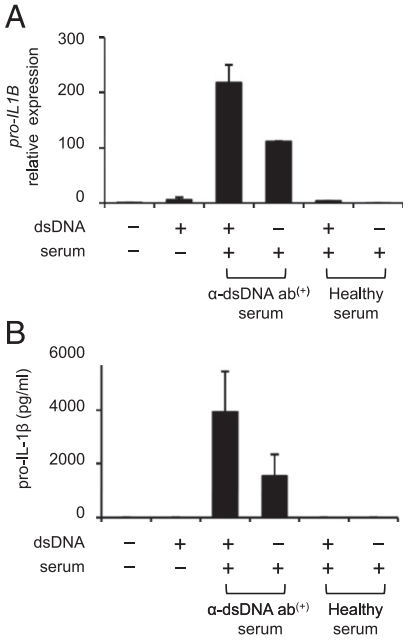

FIGURE 3. Self dsDNA induces pro–IL-1β in human monocytes in the presence of anti–dsDNA Ab^+ serum. (**A** and **B**) Monocytes from healthy donors were incubated for 6 (qPCR) or 10 h (ELISA) with dsDNA (5 μg/ml) in the presence or absence of healthy or anti–dsDNA Ab^+ serum (5% final concentration). (A) Pro-*IL1B* gene expression was measured by qPCR. (B) Intracellular pro–IL-1β was measured using cell lysates by ELISA. Bars and error bars indicate mean and SEM, respectively [$n = 2$ and 3 independent experiments for (A) and (B), respectively].

The production of IL-1β by human monocytes in response to self dsDNA and anti–dsDNA Abs requires caspase-1 and NLRP3

We investigated whether self dsDNA could activate caspase-1 in human monocytes in the presence or absence of anti–dsDNA Ab^+ serum. Increased caspase-1 activation was found in monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum but not in the cells treated with dsDNA alone or a combination of dsDNA and healthy serum (Fig. 5A, Supplemental Fig. 2B). Although increased caspase-1 activation was detected in monocytes treated with anti–dsDNA Ab^+ serum alone, the levels of such activation were lower than those in monocytes treated additionally with dsDNA. Similar findings were observed when monocytes were treated with IgG purified from anti–dsDNA Ab^+ serum (Supplemental Fig. 2D). Furthermore, caspase-1 inhibitor significantly suppressed IL-1β production from monocytes treated with dsDNA along with anti–dsDNA Ab^+ serum (Fig. 5B) or anti–dsDNA Ab^+ serum alone (Supplemental Fig. 3). Of interest, we noticed increased *NLRP* gene expression in monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum (Fig. 5C), suggesting the role of the NLRP3 inflammasome in activating caspase-1. To determine whether this caspase-1 activation was dependent on the NLRP3 inflammasome, we knocked down NLRP3 expression in human primary monocytes (Supplemental Fig. 4). *NLRP3* knockdown substantially reduced IL-1β production from monocytes stimulated with a combination of dsDNA and anti–dsDNA Ab^+ serum (Fig. 5D). These findings indicate that the NLRP3 inflammasome plays an important role in producing IL-1β from human monocytes in response to self dsDNA and anti–dsDNA Abs.

ROS synthesis and potassium efflux are involved in producing IL-1β from human monocytes in response to self dsDNA and anti–dsDNA Abs

ROS has been suggested as a key mediator in the activation of the NLRP3 inflammasome (7). We measured intracellular ROS gen-

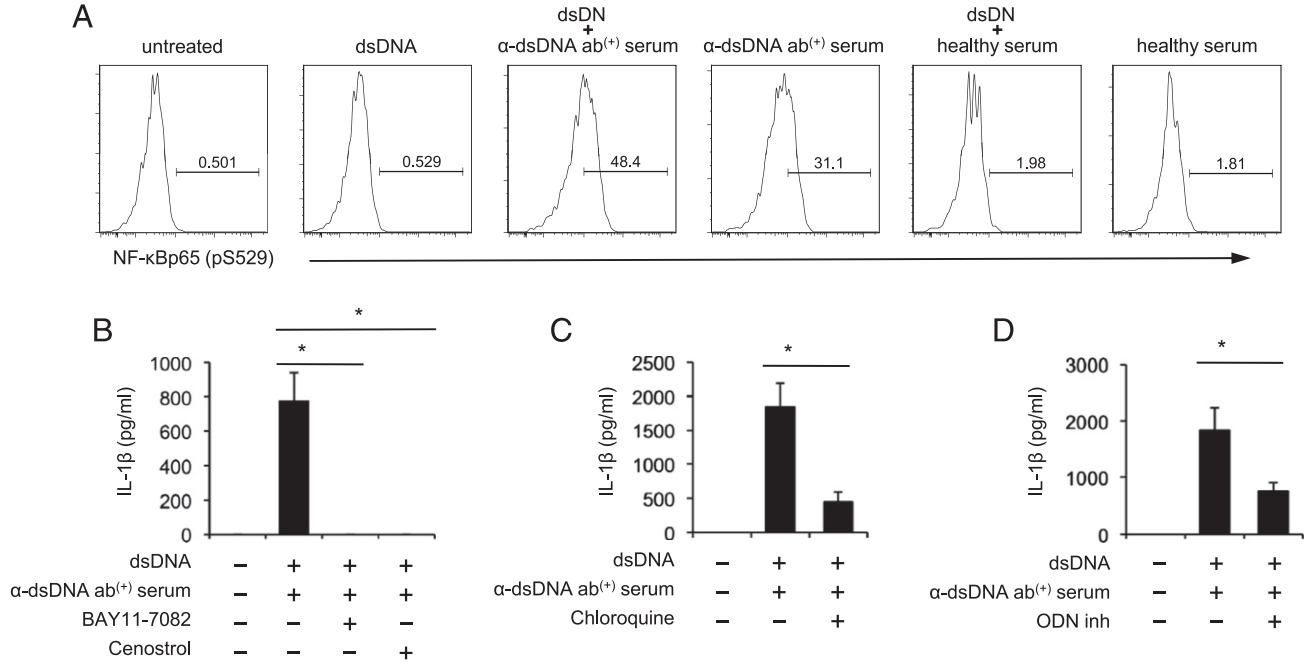

FIGURE 4. The production of IL-1β from human monocytes by self dsDNA and anti–dsDNA Ab^+ serum is dependent on TLR9 and NF-κB activation. (**A**–**D**) Monocytes were purified from healthy donors for the following experiments. (A) Monocytes were incubated for 4 h with dsDNA in the presence or absence of healthy or anti–dsDNA Ab^+ serum. NF-κB activation (phosphorylation) was determined by flow cytometry. Numbers in histograms indicate the frequency (percentage) of cells stained for phosphorylated NF-κB (pS529). (B–D) Monocytes were incubated for 18 h with dsDNA and anti–dsDNA Ab^+ serum in the presence or absence of the NF-κB inhibitors (B, 5 μM Bay11-7082 and 5 μM celastrol), chloroquine (C, 5 μg/ml), or inhibitory nucleic acid sequence for TLR9 (D, 5 μM oligodeoxynucleotide). IL-1β in cell culture supernatants was measured by ELISA. Representative data from four independent experiments (A). Bars and error bars indicate mean and SEM, respectively [n = 4, 7, and 8 for (B), (C), and (D), respectively]. *$p < 0.05$.

eration in human monocytes in response to self dsDNA and/or anti–dsDNA Ab^+ serum. The generation of ROS was increased in monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum compared with the cells treated with dsDNA or a combination of dsDNA and healthy serum (Fig. 6A, Supplemental Fig. 2C). However, monocytes treated with dsDNA or healthy serum with or without dsDNA could not increase ROS synthesis. Although ROS generation was detected in monocytes treated with anti–dsDNA Ab^+ serum alone, the levels of ROS generation were lower in these cells than those in monocytes treated additionally with dsDNA (Fig. 6A). We also measured ROS in monocytes treated with purified IgG from anti–dsDNA Ab^+ serum in the presence of dsDNA. The increased generation of ROS was found in such treated monocytes (Supplemental Fig. 2E). Furthermore, blocking the generation of ROS with the NADPH oxidase inhibitor DPI significantly decreased IL-1β production (Fig. 6B, Supplemental Fig. 3). In addition to ROS, potassium efflux has been suggested as an activator for the NLRP3 inflammasome (7). To study the role of potassium efflux in the IL-1β secretion, we suppressed potassium efflux by adding potassium to cell culture media. Indeed, increasing extracellular potassium significantly reduced IL-1β production from monocytes treated with the combination of dsDNA and anti–dsDNA Ab^+ serum (Fig. 6C). These results suggest the involvement of ROS and potassium efflux in inducing IL-1β production from human monocytes in response to dsDNA and anti–dsDNA Abs by activating the NLRP3 inflammasome.

IL-17 production from $CD4^+$ T cells is promoted in the presence of culture supernatant from monocytes treated with dsDNA and its autoantibody$^+$ serum

IL-1β promotes the differentiation of IL-17–producing Th17 cells (38, 39). Memory $CD4^+$ T cells with the expression of IL-1RI potently produced IL-17, and IL-1β increased such cytokine production (35). A role for IL-17 in lupus pathogenesis has been suggested by recent studies showing enhanced Ab production from B cells by IL-17 (27) and increased Th17 cell response in lupus patients (27, 29, 30). However, the mechanism for the latter finding is unknown. Thus, we determined whether monocytes stimulated with a combination of dsDNA and anti–dsDNA Ab^+ serum could promote IL-17 production from IL-$1R1^+$ memory $CD4^+$ T cells through IL-1β production. Indeed, culture supernatant from such treated monocytes enhanced IL-17 production from IL-$1R1^+$ memory $CD4^+$ T cells, which was blocked by adding IL-1 receptor antagonist to the supernatant (Fig. 7).

Discussion

Armed with the germline-encoded PRRs that recognize PAMPs, innate immune cells such as monocytes provide the first line of host defense against microorganisms (1). In addition to PAMPs, some PRRs can be triggered by self molecules, called damage-associated molecular patterns, released in the setting of cell death (2). Inflammasomes are multimeric protein complexes with the capacity to activate caspase-1, which is required to mature and secrete the proinflammatory cytokine IL-1β by cleaving pro–IL-1β into IL-1β. Different types of inflammasomes contain distinct PRRs that are responsible for the activation of the inflammasomes by recognizing the ligands including nucleic acids (5, 6, 18, 21, 40–43). dsDNA is a molecular target of autoimmune responses in lupus that affects multiple organs, including the skin and joints (8). In fact, anti–dsDNA Abs and circulating dsDNA/anti–dsDNA immune complexes are frequently found in lupus patients (9, 10). In this study, we showed that self dsDNA could induce IL-1β production from human monocytes, a major cellular source of IL-1β, in the presence of anti–dsDNA Abs by activating the TLR9 pathway and caspase-1–containing NLRP3 inflammasome. ROS

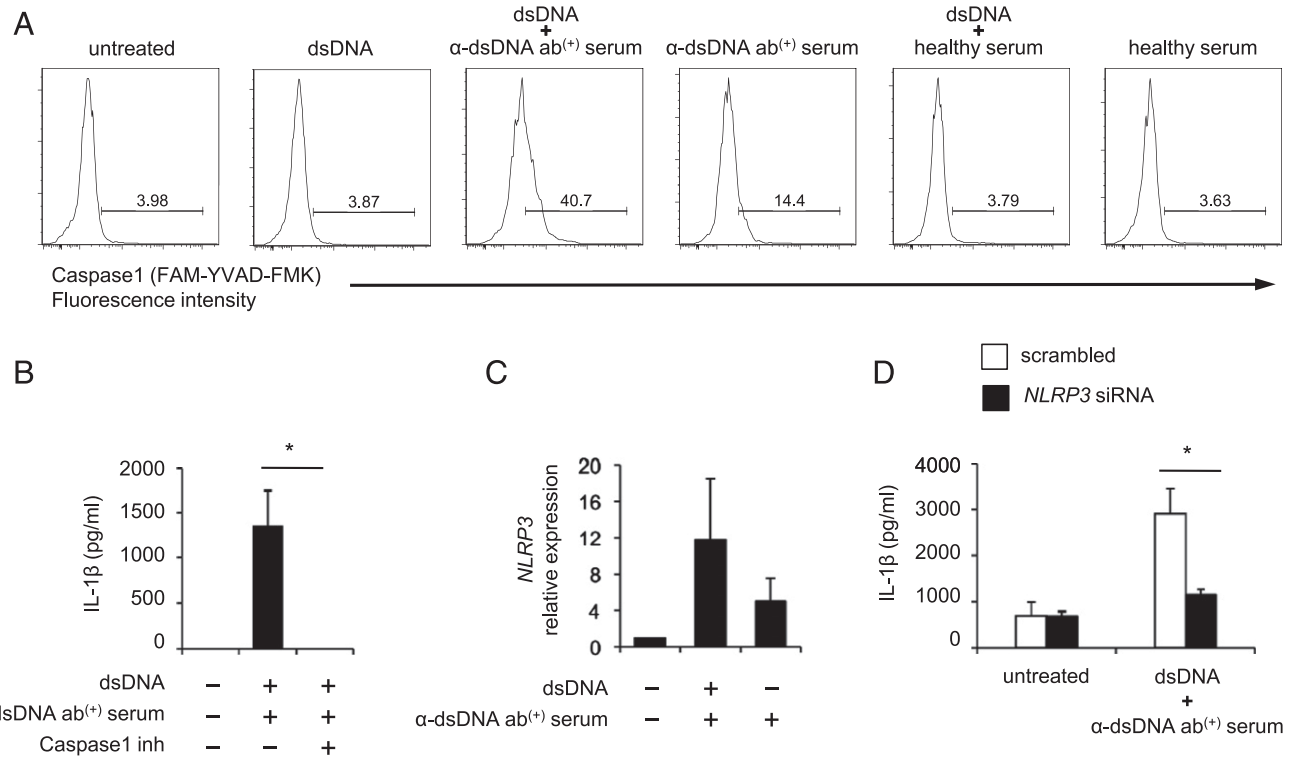

FIGURE 5. Caspase-1 and NLRP3 are involved in the production of IL-1β from human monocytes in response to self dsDNA and anti–dsDNA$^+$ serum. (**A**) Monocytes from a healthy donor were incubated for 7 h with dsDNA in the presence or absence of healthy or anti–dsDNA Ab$^+$ serum. Active capspase-1 was measured using flow cytometry. Numbers in histograms indicate the frequency (percentage) of cells positive for active caspase-1. (**B**) IL-1β ELISA of culture supernatants from monocytes incubated for 18 h with dsDNA and anti–dsDNA Ab$^+$ serum in the presence or absence of caspase-1 inhibitor (10 μM) (n = 4). (**C**) Measuring the *NLRP3* gene expression in human monocytes incubated for 6 h in the presence or absence of anti–dsDNA Ab$^+$ serum or a combination of dsDNA and anti–dsDNA Ab$^+$ serum by qPCR (n = 2). (**D**) IL-1β ELISA of culture supernatants from monocytes nucleofected with scrambled or *NLRP3*-specific siRNA followed by the incubation for 18 h with dsDNA and anti–dsDNA Ab$^+$ serum (n = 6). Representative data from four independent experiments (A). Bars and error bars indicate mean and SEM, respectively. *$p < 0.05$.

and K^+ efflux were likely responsible for the activation of the NLRP3 inflammasome in monocytes treated with self dsDNA and anti–dsDNA Abs. Indeed, knocking down the *NLRP3* or inhibiting ROS synthesis, K^+ efflux, caspase-1, or TLR9 pathway decreased IL-1β production from such treated monocytes. Additionally, Th17 cell response, which is known to be increased in lupus patients, was enhanced by supernatants from monocytes treated with dsDNA and anti–dsDNA Ab$^+$ serum in an IL-1β–dependent manner. These findings elucidate a possible role of IL-1β and NLRP3 inflammasome in the pathogenesis of lupus as well as a mechanism involved in the production of such cytokine from monocytes in response to self dsDNA and its autoantibodies.

We showed that self dsDNA could stimulate human monocytes and induce IL-1β production in the presence of anti–dsDNA Abs. The immune stimulatory property of dsDNA was reported in B cells, pDCs, and keratinocytes (10, 13–17). In the presence of anti–dsDNA Abs, self dsDNA stimulated B cells and pDCs dependent on TLR9, leading to increased Ab and IFN-α production, respectively (10, 13, 14, 17). In our study, treating dsDNA or anti–dsDNA Ab$^+$ serum with DNase suppressed IL-1β production from human monocytes, further supporting the immune stimulatory property of this molecule. Indeed, mice deficient of DNase I developed a lupus-like disease (44). Also, lupus patients with a nonsense mutation in one allele of DNase I had increased disease activity with reduced DNase I activity (45).

Previous studies showed the activation of AIM2 inflammasome by self and non-self dsDNA in innate immune cells and keratinocytes upon infection with virus or transfection with plasmid or host DNA (5, 6, 18–20). Additionally, the NLRP3 inflammasome was involved in producing IL-1β from THP-1 cells and murine macrophages infected with adenovirus, which is a nonenveloped DNA virus (21). DNA released from apoptotic hepatocytes induced pro-*ilb* gene expression in hepatocytes and liver sinusoidal endothelial cells from mice in a TLR9-dependent manner (46). In our study, knocking down NLRP3 or inhibiting TLR9 suppressed IL-1β production from monocytes in response to self dsDNA and anti–dsDNA Ab$^+$ serum. This clearly supports the role of the NLRP3 inflammasome and TLR9 in activating monocytes when self dsDNAs are internalized into the cells dependent on its Abs. Such an event is likely linked to the generation of ROS and K^+ efflux, known NLRP3 inflammasome activators, as inhibiting ROS synthesis or K^+ efflux decreased IL-1β production. In the generation of ROS, the endosome appears to be involved in that the endosomal inhibitor chloroquine reduced ROS synthesis by monocytes treated with dsDNA and anti–dsDNA Ab$^+$ serum (Supplemental Fig. 1C). Of interest, we noticed variability in the capacity to induce IL-1β production from dsDNA-treated monocytes among different anti–dsDNA Ab$^+$ sera (5% final concentration), ranging from below the level of detection to nanograms. Such variability could be related to the binding affinity of anti–dsDNA Abs to dsDNA (11, 12). We found that self dsDNA alone could not activate NF-κB or induce IL-1β production from human monocytes unless anti–dsDNA Abs were present. This finding could explain why inflammation may not occur in healthy people who do not have anti–dsDNA Abs even though dsDNA could be released from dying cells. The NLRP3 inflammasome with active caspase-1 is also involved in cleaving pro–IL-18 to the active form IL-18 (2). Similarly to IL-1β, we detected IL-18 production from

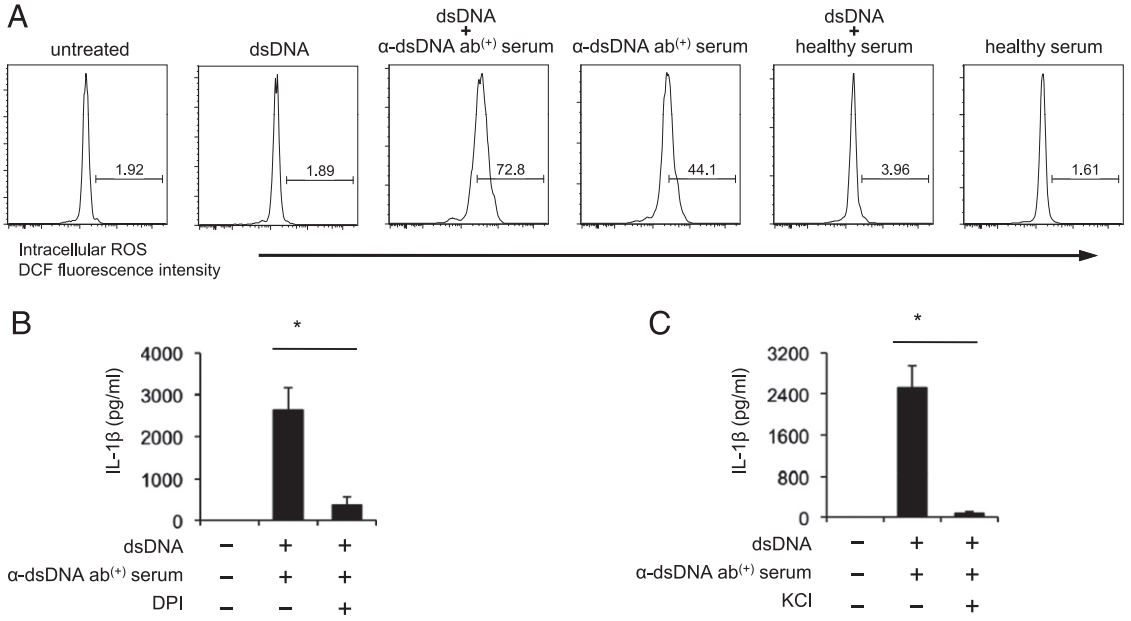

FIGURE 6. The production of IL-1β from human monocytes in response to self dsDNA and anti–dsDNA Ab^+ serum requires ROS synthesis and K^+ efflux. (**A**) Flow cytometric analysis of ROS in monocytes stimulated for 4 h with dsDNA in the presence or absence of healthy or anti–dsDNA Ab^+ serum. (**B** and **C**) IL-1β ELISA of culture supernatants from monocytes incubated for 18 h with dsDNA and anti–dsDNA Ab^+ serum in the presence or absence of the ROS inhibitor 50 μM DPI (B) or 100 mM KCl (C). Representative data from three independent experiments (A). Bars and error bars indicate mean and SEM, respectively [n = 5 and 7 for (B) and (C), respectively]. *$p < 0.05$.

monocytes treated with a combination of dsDNA and anti–dsDNA Ab^+ serum or anti–dsDNA Ab^+ serum alone, although the combination induced much higher levels of IL-18 production than did the Ab-positive serum alone (Supplemental Fig. 2F).

IL-1β is a potent proinflammatory cytokine that can induce the expression of other proinflammatory molecules such as IL-6 and cyclooxygenase-2 (47). Previous studies reported increased IL-1β gene or protein expression in PBMCs or skin lesions of lupus patients as well as in the kidneys of MRL-*lpr* and New Zealand Black/White mice with lupus-like disease (22–26). In an experimental mouse model of lupus induced by injecting anti–dsDNA mAbs, nephritis was less severe in IL-1β–deficient mice compared with wild-type mice (48). In fact, decreased arthritis was noticed in patients with SLE who received rIL-1R antagonist anakinra (49). In a recent study, we also demonstrated IL-1β production from human monocytes in response to the autoantigen U1-small nuclear ribonucleoprotein and anti–U1-small nuclear ribonucleoprotein Abs, which are found in lupus patients (50). These findings suggest an implication of IL-1β in the pathogenesis of lupus. Of interest, IL-1β is known to promote the development of Th17 cells that produce the proinflammatory cytokine IL-17. Patients with SLE had an increased frequency of Th17 cells in the peripheral blood that correlated with disease activity (28, 30). Additionally, IL-17–producing T cells were found in the nephritis tissue from patients with SLE and lupus-prone mice (29, 51). Thus, it is conceivable that IL-1β produced from monocytes in response to a combination of dsDNA and anti–dsDNA Abs could be a factor contributing to increased Th17 cell response in lupus. Indeed, in our study, IL-17 production was increased in human $CD4^+$ T cells with the expression of IL-1RI (35) by adding cell culture supernatant from monocytes stimulated with dsDNA and anti–dsDNA Ab^+ serum. Such an increase was blocked by IL-1R antagonist.

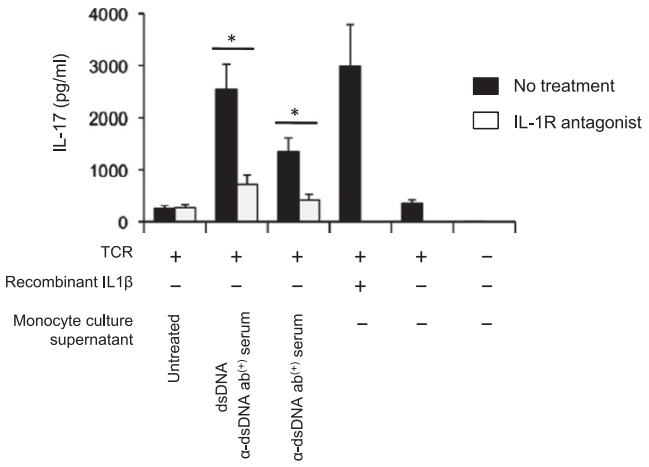

FIGURE 7. Cell culture supernatant from monocytes treated with a combination of self dsDNA and anti–dsDNA Ab^+ serum promotes IL-17 production from IL-1RI–positive memory $CD4^+$ T cells in an IL-1β–dependent manner. ELISA of IL-17 production from sorted human $IL\text{-}1RI^+$ memory $CD4^+$ T cells that were stimulated for 5 d with anti–CD3 and anti–CD28 Ab-coated beads in 80% culture medium with 20% of supernatants from monocytes treated for 18 h with self dsDNA in the presence or absence of anti–dsDNA Ab^+ serum. Some $IL\text{-}1RI^+$ memory $CD4^+$ T cells were incubated with culture medium alone or medium with anti–CD3 and anti–CD28 Ab-coated beads in the presence or absence of rIL-1β (20 ng/ml). IL-1R antagonist (100 ng/ml) was added on days 0 and 2. Bars and error bars indicate mean and SEM, respectively (n = 5). +, Presence of treatment; −, absence of treatment. *$p < 0.05$.

Taken together, we found that self dsDNA, a molecular target of autoimmune responses in lupus, could induce IL-1β production from human monocytes in the presence of anti–dsDNA Abs. This phenomenon occurred by activating the TLR9 pathway and caspase-1–containing NLRP3 inflammasome that cleaves pro–IL-1β into mature IL-1β. The NLRP3 inflammasome activation by the self dsDNA and its autoantibodies was likely driven by ROS synthesis and K^+ efflux. Indeed, the production of IL-1β from such treated monocytes was suppressed by knocking down the *NLRP3* or inhibiting ROS synthesis, K^+ efflux, caspase-1, or TLR9 pathway. Lastly, Th17 cell response was enhanced by supernatants

from monocytes treated with dsDNA and anti–dsDNA Ab^+ serum in an IL-1β–dependent manner. These findings provide new insights in lupus pathogenesis by linking the target autoantigen dsDNA to the NLRP3 inflammasome, IL-1β, and IL-17 production. Our results also offer a scientific rationale for the therapeutic possibility of inhibiting this molecular pathway in human lupus.

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Disclosures

The authors have no financial conflicts of interest.

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